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EXTRACTION AND CHARACTERIZATION OF PLANT EXTRACTS AND EVALUATION OF THEIR SYNERGISTIC EFFECT WITH DIFFERENT ANTIBIOTICS ON *S.aureus*, *K. peumoniae*, and *P.aeruginosa*

SRIVASTAVA ML[1] AND SARFARAZ MA[2*]

1: Department of Botany, Jai Prakash University, Chapra (Bihar)

2: Head, University Department of Botany, Jai Prakash University, Chapra (Bihar)

***Corresponding Author: MD. Sarfaraz Ahmad: E Mail: mdsarfarazahmad786@gmail.com;**

Mobile: +91-9431275300

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ABSTRACT

The growth of antibiotic-resistant microbes is increasing at an alarming rate and hence its creating difficulties in the treatment of various infectious diseases and hence there is an urgent need for new and efficient antimicrobial agents that could help to combat the problem easily. The plant-derived compounds have shown to act as the potential compounds that have good antimicrobial compounds as well as they can modify the activity of standard drugs when administered in combinations. In the present study, the methanolic extract was prepared for both the selected plants that are *Tinospora cordifolia* and *Eclipta prostrata*, the methanolic extracts were then analyzed for the presence of potentially bioactive compounds followed by the determination of their antimicrobial property against *K. pneumoniae, P. aeruginosa* and *S. aureus*. Then the synergistic property of the extracts with four different antibiotics was determined by obtaining the MIC value for their individual and combined effects. The MIC value was used for the calculation of Fractional inhibitory concentration (FIC) followed by the calculation of fractional inhibitory concentration index (FICI) value that helped to interpret the type of interaction possessed by the plant extract and antibiotic combinations. The results obtained from the study clearly depicted that both the plant extracts have shown to be a potential

antibacterial agent as they are rich in bioactive constituents. The FICI value has shown that the better and maximum synergistic property has been shown by the methanolic extract of *T. cordifolia* while the methanolic extract of *E. prostrata* also showed it for some combinations. The study concludes that these plants could be considered for the formulations of new antimicrobial drugs that could impart a better and effective activity against the Multi-drug resistance pathogenic microbes.

Keywords: Bioactive compounds, Pathogenic bacteria, Antimicrobial property, Synergistic activity

INTRODUCTION

One of the serious problems that are leading to the treatment failures of the infections and increasing the mortality rate worldwide is the steady increase of multi-drug resistance in the pathogenic organisms **[1]**. The need of the hour is to develop new and potential antimicrobial compounds or drugs that could block the resistance mechanisms and could help to improve the treatment strategies to eradicate the resistant strains. The bacteria possess a remarkable strategy to adapt to its adverse environmental conditions and hence survive at the clinically relevant concentrations of the existing antibiotics. This process of development of antibiotic-resistant is accelerating at an alarming rate due to the misuse of antibiotics. The antibiotics induce several genetic changes in the bacteria that lead to the selection of resistant strains **[2]**. The ability to acquire the resistance is enhanced by the process like rapid gene mutation and horizontal gene transfer. The use of two or more antibacterial agents from either the same or different origin for the treatment is one of the important strategies to overcome the multidrug resistance problem **[3]**.

The plants are the reservoir for a whole different series of compounds that are not significantly considered as the primary metabolites but have a remarkable effect to plant pathogenic microorganisms and also impart the medicinal or pharmacological properties to the plant. These compounds are collectively considered as the secondary metabolites and they are responsible for the unique plant characteristic features like color, scent, and flavor and also imparts the biological and pharmacological properties **[4]**. These bioactive compounds are structurally diverse compounds classified as the phenolic compounds, alkaloids, and terpenes. These compounds are easily extracted from the plant materials as a solvent extract or in the form of essential oils.In the recent scenario, the antimicrobial

secondary metabolites from plants have been shown to carry the synergistic activity even when they do not possess the antimicrobial property on their own. But they enhance the properties and efficiency of the standard drugs when combined **[5]**. Some of the general mechanisms of their action in the context that are acceptable include the inhibition of protective enzymes, biochemical pathways, or the use of membranotropic agents that would improve their diffusion across the membrane **[6]**. Such kind of study that involves the combined formulation of standard drugs with the plant extracts is termed as Phytotherapy which is a potential and significantly important way with the synergistic interactions that imparts the increased efficiency, reduces the undesirable effects, and also provides stability to the compounds **[7]**.

T. cordifolia is a member of the family Menispermaceae and is also known as Guduchi or Amrita while also called as Giloya in Hindi **[8]**. This plant is traditionally used in the preparations of ayurvedic medicines and also carries some therapeutic properties **[9]** like it is employed in the treatment of inflammation, allergy, diabetes, skin diseases, and the urinary disorders **[10]**. The starch obtained from this plant serves as the household remedy for chronic fever it relieves the burning sensation and increases the appetite. The plant possesses such potential pharmacological properties due to the presence of some bioactive constituents like glycosides, diterpenoid lactones, sesquiterpenoid, phenolics, aliphatic compounds, and essential oils **[11]**.*Eclipta prostrata* is a member of the family Asteraceae and it is very commonly found in the tropical and subtropical regions. This herb is used in the treatment of hepatitis in India and the treatment against the snake venom in brazil. This medicinal plant is considered for the treatment of hyperlipidemia, hepatic disorders **[12]** and also in the inflammatory and skin diseases **[13]**. The current investigation aims to determine the antibacterial potential of the plant extracts as well as to determine the synergistic activity of these extracts with the antibiotics to derive the interpretations for their positive impact on the activity potential and also give a view for the development of new antibacterial agents.

MATERIALS AND METHODOLOGY

Preparation of plant extracts

Fresh leaves sample of *Tinospora cordifolia* and *Eclipta prostrata* were collected directly from the organic producers from the local area of Gopalganj district, Bihar. The samples were immediately brought to the lab

and washed with distilled water thrice to completely remove the dirt and dust. The samples were freeze-dried and eventually grounded into the fine powder using a blender and stored in bottles until the extraction process. The extracts from both samples were prepared by implicating the solid-liquid extraction method, for which 2gm of the finely grounded powder of each sample separately was mixed with 100 ml of Methanol solvent and added into a screw cap flask. The flasks were kept in the water bath at 70ºC and were stirred periodically for 30 minutes. The extracts obtained were filtered through the No. 1 Watman^{TM} filter paper and also centrifuged at 8500g for 5 minutes to collect the supernatant in fresh bottles. Both methanolic extracts were then subjected to complete evaporation by rotatory evaporator at 40ºC. The dried extract finally obtained were dissolved in 10% DMSO to prepare the final concentration of 5mg/ml for each extract **[14]**.

Characterization of plant extracts

Qualitative Analysis

The qualitative screening for the presence of phytochemical compounds in both plant extracts was done by the standard operating protocols used for the detection. The extracts were used for the determination of different phytochemical compounds like Alkaloids-Wagner's test **[15]**, Flavonoids-Alkaline reagent test**[16]**, Phenols- Ferric chloride test **[17]**, Tannins-Braymer's test **[18]**, Terpenoids-Salkowkis test **[19]** (Sharma *et al*., 2012), Glycosides-Keller kelliani test **[20]**, Protein-Ninhydrin test**[21]**. All these tests were performed for both extracts separately.

Quantitative Analysis

The quantitative analysis of some prominent phytochemical compounds was performed using spectrophotometric quantification methods. For estimation of total flavonoids content 0.5ml of the plant, the extract was mixed with an equal volume of 2% aluminum chloride solution and the mixture was allowed to stand for 1 hour at RT and then optical density was measured at 420nm using the Systronic UV-Visible Double Beam Spectrophotometer 2202 **[22]**. While the total phenolic content was determined by the Folin-Ciocalteu method, where 0.5ml of the extract was mixed with 5ml of Folin's reagent and 4ml of 7.5% sodium carbonate solution. The content was vortexed for 10-15 seconds and then allowed to stand for 30 minutes at 40ºC then optical density was measured at 765nm for Systronic UV-Visible Double Beam Spectrophotometer 2202 **[23]**. The final concentration in mg/ml was

determined by using the standard curve of the respective compound.

Antibacterial Assay

The antibacterial assessment of both methanolic plant extracts and the selected antibiotics (Chloramphenicol, Ciprofloxacin, Tetracycline, and Erythromycin) was done by two methods that are Agar well diffusion method and minimum inhibitory concentration (MIC) Assay. For the first assay the selected bacterial culture was inoculated in the LB broth and incubated at 37ºC for 3 hours and their turbidity was maintained at 0.5 MacFarland's index using PBS. A bacterial lawn was spread over the MHA plates using the broth and the plates were punched to form 6mm wells that were later loaded with 20 microliters of plant extract and antibiotic separately. The plates were incubated at 37ºC for 18 hours and then Zone of inhibition (ZOI) in mm was recorded **[24]**. For the determination of the MICs Micro broth dilution method using MH broth media was performed. The concentration range for MIC determination for the extract was 50µg/ml to 5000 µg/ml while for the antibiotic is 0.224µg/ml to 500µg/ml, 0.005µg/ml to 5µg/ml and 0.049µg/ml to 50µg/ml for Chloramphenicol and Tetracycline, Ciprofloxacin, Erythromycin respectively. For the assay sterile 96 well plates with flat bottom were loaded with 100µl of two-fold dilution of the extract and antibiotic separately and the starting bacterial inoculum was set at $1.5*10^5$ CFU/ml. The wells containing only the bacterial culture were treated as control and the plates were incubated at 37ºC. The highest dilution of the antibiotic and extract that showed no turbidity or growth was considered as the MIC of that respective extract or antibiotic **[25]**.

Synergistic Antimicrobial Assay

The interaction between methanolic extract of *Tinospora cordifolia* and *Eclipta prostrata* with the selected antibiotics that is Chloramphenicol, Ciprofloxacin, Tetracycline, and Erythromycin was determined by the checkerboard titration method using MH broth medium in microtiter plates as described previously **[26, 27]**. The concentration of both the extract ranged from 5000µg/ml to 50µg/ml and for the antibiotic it was taken as per their MIC value. The interaction between extract and antibiotic was determined as the Fractional Inhibitory Concentration (FIC) value, which was calculated as the MIC of the agent in combination divided by the MIC of agent individually, i.e., FIC (extract) = MIC of extract in combination / MIC of extract alone. The final result interpretation was

made based on the value of Fractional inhibitory concentration index (FICI) that is calculated as the sum of FIC value of both agents and the result obtained are followed as FICI value < 0.5 synergy; 0.5 to 1 additive effect; 1–2 indifferent or no effect; and >2 antagonism **[28, 29]**.

RESULTS AND DISCUSSION

After the final step, the methanolic plant extract of both *Tinospora cordifolia* and *Eclipta prostrata* were obtained as the dry powder, the powder was dissolved in DMSO to prepare the solution of each extract as per 5mg/ml concentration. Then immediately after this, both extracts were used for the qualitative phytochemical analysis. This qualitative analysis showed that both methanolic extracts are rich in potential bioactive compounds that are eventually responsible for their medicinal properties and health benefits. Both extracts showed the presence of alkaloids, flavonoids, terpenoids, glycosides, and protein while tannins and phenols were present only in the extract of *Eclipta prostrata* and *Tinospora cordifolia* respectively **(Table 1)**. Then the extracts were undertaken for the quantification of the prominent bioactive compound that is phenols and flavonoids. The quantification study showed that the methanolic extract of *Tinospora cordifolia*is rich in both bioactive compounds than the methanolic extract of *Eclipta prostrata* having flavonoids as (10.8mg/ml and 7.5mg/ml) and phenols as (8.6mg/ml and 5.4mg/ml).

The susceptibility study conducted for the plant extracts and the selected antibiotic by the agar well diffusion assay clearly showed that both the methanolic plant extracts have potential antibacterial activity against all three selected microorganisms., *K. pneumoiae, P. aeruginosa*, and *S. aureus*. The antibacterial property of both extracts was found to be better than the antibiotics undertaken in the study, as the result interpretation for the antibiotic clearly shows that they were least active against the selected microbes and most of them were resistant at the used concentration **(Table 2)**.

In order to determine the synergistic activity of the methanolic extracts, the Minimum Inhibitory Concentration (MIC) value was determined for both the extracts and all the selected antibiotic individually and in combinations also against all three bacterial isolates. The resultant MIC value **(Table 3)** clearly shows that the antibiotics had a higher MIC value individually while the combination of them with extracts has decreased the MIC concentration drastically. It depicts that the interaction between the antibiotics and the methanolic extracts good

produce a good impact on the antibacterial activity of each other.

Based on the MIC values obtained for the individual effect and the combined effect for each combination developed, the value for fractional inhibitory concentration (FIC) was calculated by the previously mentioned formulae. The value of FIC for both extract and its combining antibiotic was summed up to obtain the values for fractional inhibitory concentration index (FICI) and the basis of the FICI value scale the results for each interpretation was determined **(Table 4 and 5)**. The resultant data shows that the methanolic extract of *Tinospora cordifolia* has the good and maximum number of synergistic activity relation with 3 out of 4 of the selected antibiotics against *K. pneumonia* while the same extract had no synergistic activity against *S. aureus* with any combinations. The methanolic extract of *Eclipta prostrata* showed the minimum synergistic activity against all the three isolates with some antibiotic combination like with tetracycline against *K. pneumoiae* and with chloramphenicol against *P. aeruginosa* and *S. aureus* with strong synergy values of 0.404, 0.380, and 0.343 respectively as the MIC of the antibiotic decreased drastically. All the synergy effects shown by both extracts are of very good impact as a maximum value decrement for the MIC is observed in all the cases.

Table 1: Showing the result for the phytochemical analysis of a methanolic extract of *Tinospora cordifolia* and *Eclipta prostrata*, (+) present; (-) absent

S. No.	Phytochemical compound	Tinospora cordifolia	Eclipta prostrata
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Phenols	+	-
4	Tannins	-	+
5	Terpenoids	+	+
6	Glycosides	+	+
7	Protein	+	+

Table 2: Showing the results for Antibacterial assay of the extracts and antibiotics against selected bacterial species by agar well diffusion method where *R – Resistant *I – Intermediate *S- Susceptible

S. No.	Antibacterial agents / Conc.	Zone of inhibition mm					
		K. pneumoniae		*P. aeruginosa*		*S. aureus*	
1	*T. cordifolia* (5mg/ml)	23	-	22	-	23	-
2	*E. prostrata* (5mg/ml)	21	-	24	-	22	-
3	Chloramphenicol (30µg/ml)	12	R	15	I	18	S
4	Ciprofloxacin (5µg/ml)	16	I	15	R	20	I
5	Tetracycline (30 µg/ml)	17	I	14	R	19	S
6	Erythromycin(15 µg/ml)	13	R	10	R	17	I

Table 3: Showing the value of the minimum inhibitory concentration for the antibiotic, extracts, and their combinations

S. No.	Antibacterial agents	Minimum Inhibitory concentration (MIC)		
		Organisms		
		K. pneumoniae	*P. aeruginosa*	*S. aureus*
1	TET µg/ml	260	200	300
2	ERY µg/ml	45	35	30
3	CHL µg/ml	350	250	200
4	CIP µg/ml	5.0	4.0	4.0
5	TC mg/ml	2.5	3.5	2.15
6	EP mg/ml	2.0	2.5	3.15
7	TC/TET	0.62/35	0.75/40	0.90/50
8	TC/ERY	0.85/20	0.60/30	0.70/45
9	TC/CHL	0.62/32	0.90/50	1.0/60
10	TC/CIP	0.75/0.5	0.75/2.0	0.62/2.5
11	EP/TET	0.50/40	0.60/55	0.40/45
12	EP/ERY	0.62/25	0.55/15	0.50/15
13	EP/CHL	0.80/70	0.45/50	0.45/40
14	EP/CIP	1.0/3.0	0.45/2.5	0.40/1.5

TET-Tetracycline, ERY- Erythromycin, CHL- Chloramphenicol, CIP- Ciprofloxacin, TC- *Tinospora cordifolia* and EP- *Eclipta prostrate

Table 4: Showing the antimicrobial synergistic activity of the methanolic extract of *Tinospora cordifolia* with different antibiotics

Organism	Combination	FIC value		FICI	Interpretation
K. pneumoniae	TC/TET	TC	TET	0.383	Synergistic
		0.248	0.135		
	TC/ERY	TC	ERY	0.784	Additive
		0.340	0.444		
	TC/CHL	TC	CHL	0.339	Synergistic
		0.248	0.091		
	TC/CIP	TC	CIP	0.400	Synergistic
		0.300	0.100		
P. aeruginosa	TC/TET	TC	TET	0.414	Synergistic
		0.214	0.200		
	TC/ERY	TC	ERY	1.029	Intermediatory
		0.171	0.857		
	TC/CHL	TC	CHL	0.457	Synergistic
		0.257	0.200		
	TC/CIP	TC	CIP	0.714	Additive
		0.214	0.500		
S. aureus	TC/TET	TC	TET	0.585	Additive
		0.419	0.167		
	TC/ERY	TC	ERY	1.826	Intermediatory
		0.326	1.500		
	TC/CHL	TC	CHL	0.765	Additive
		0.465	0.300		
	TC/CIP	TC	CIP	0.913	Additive
		0.288	0.625		

TET-Tetracycline, ERY- Erythromycin, CHL- Chloramphenicol, CIP- Ciprofloxacin, TC- *Tinospora cordifolia* and EP- *Eclipta prostrate

Table 5: Showing the antimicrobial synergistic activity of the methanolic extract of *Eclipta prostrata* with different antibiotics

Organism	Combination	FIC value		FICI	Interpretation
K. pneumoniae	EP/TET	EP	TET	0.404	Synergistic
		0.250	0.154		
	EP/ERY	EP	ERY	0.866	Additive
		0.310	0.556		
	EP/CHL	EP	CHL	0.600	Additive
		0.400	0.200		
	EP/CIP	EP	CIP	1.100	Intermediatory
		0.500	0.600		
P. aeruginosa	EP/TET	EP	TET	0.515	Additive
		0.240	0.275		
	EP/ERY	EP	ERY	0.649	Intermediatory
		0.220	0.429		
	EP/CHL	EP	CHL	0.380	Synergistic
		0.180	0.200		
	EP/CIP	EP	CIP	0.805	Additive
		0.180	0.625		
S. aureus	EP/TET	EP	TET	0.277	Synergistic
		0.127	0.150		
	EP/ERY	EP	ERY	0.659	Additive
		0.159	0.500		
	EP/CHL	EP	CHL	0.343	Synergistic
		0.143	0.200		
	EP/CIP	EP	CIP	0.502	Additive
		0.127	0.375		

TET-Tetracycline, ERY- Erythromycin, CHL- Chloramphenicol, CIP- Ciprofloxacin, TC- *Tinospora cordifolia* and EP- *Eclipta prostrata

The problem of resistance against the antimicrobial agents is growing among the prominent pathogens and the outlook for using them in the future is still uncertain. Although in the last few decades the pharmacological industries have shown their potential and produced new antimicrobial drugs but the resistance to these new drugs in bacteria has also increased a lot **[30]**. The medicinal plants that are known to date are the rich and valuable source for the new and biologically active molecules called the bioactive compounds that carry the antibacterial activity along with other biological activities. The medicinal plants carry the pharmacological activity due to the presence of polyphenolic compounds like alkaloids, flavonoids, phenols, tannins **[31]**. The qualitative analysis undertaken in the present study shows that the methanolic extracts of both the selected plant are rich in such polyphenolic compounds and this confers to their biological importance. The biological activity and importance of most of the phytochemical compounds known is determined and recorded like, tannins impart the antimicrobial activity by damaging the bacterial membrane or delaying the bacterial growth for sufficient time **[32]**, flavonoids are known to inhibit the functional roles of

DNA gyrase enzyme, carrier protein activity and function of the cytoplasmic membrane **[33]** whereas the phenolic compounds carry its biological property to the redox properties that allows it to act as the reducing agent and antioxidant agent **[34]**. The present study has shown that the methanolic extracts of *T. cordifolia* and *E. prostrata* that are rich in polyphenolic compounds have shown a very good antimicrobial property better than the antibiotics used under the study. The antimicrobial activity of the plant extracts can be attributed both as a direct effect against the bacteria or as a synergistic effect in combination with the antibiotics. The present study incorporated the organism that is considered as the environmental strains of the pathogenic organisms that often possess the problem of drug resistance in the clinical background. The major objective of the present study was to investigate the synergistic effect of the plant extracts with the antibiotic that could bring up to the interpretation that such a combination could bring the positive changes in the susceptibility of the test strains. The investigation was successful to interpret that the synergistic effect of the extracts with an antibiotic has positive variation as the combined effects decrease the MIC values. Such combinations of plant extracts and antibiotics are considered to be the potential fundamental therapy for treating various ailments **[35]**.

CONCLUSION

The synergistic activity study under the in vitro conditions of the different plant extracts have been studied against the multi-drug resistant bacteria under several scientific studies. The progress of research in such a combination study to determine the synergistic activity could lead to the development of the new antibacterial agents from the plant origin with potential properties. The present study concludes that the plant extracts considered under the study that is *Tinospora cordifolia* and *Eclipta prostrata* are potential agents that could be employed for the synergistic combination with different antibiotics and also such combination have shown be effective against the pathogenic isolates. The combination of plant extracts with the antibiotic has lowered the MIC values showing their impactful consideration for their incorporation in formulations of new drugs. As the mechanism underlying the synergistic property is still poorly known and explored, only with the exact knowledge and the strategies a standardized and effective preparation could be developed. Our findings advocate for the study to determine the

underlying mechanisms in detail and furthermore the in vivo study of such combinations in animal models should be considered to determine the toxicity and bioavailability of such combined formulations.

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Conflict-of-interest

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